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The perivascular niche microenvironment in brain tumor progression

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Glioblastoma, the most frequent and aggressive malignant brain tumor, has a very poor prognosis of approximately 1-year. The associated aggressive phenotype and therapeutic resistance of glioblastoma is postulated to be due to putative brain tumor stem-like cells (BTSC). The best hope for improved therapy lies in the ability to understand the molecular biology that controls BTSC behavior. The tumor vascular microenvironment of brain tumors has emerged as important regulators of BTSC behavior. Emerging data have identified the vascular microenvironment as home to a multitude of cell types engaged in various signaling that work collectively to foster a supportive environment for BTSCs. Characterization of the signaling pathways and intercellular communication between resident cell types in the microvascular niche of brain tumors is critical to the identification of potential BTSC-specific targets for therapy.

Introduction

Progress in the treatment of malignant brain tumors has been disappointing, particularly in regards to the treatment of high-grade gliomas (WHO grades III and IV). With current standard therapy, which includes surgical resection when feasible, radiation and temozolomide chemotherapy, patient survival is a mere 2–5 years for anaplastic glioma patients and only 12–15 months for patients with glioblastoma.[1] Studies suggest that the development and prevalent resistance of brain tumors is attributed to a small population of tumor cells within the heterogeneous tumor mass with stem-like character. These cells form tumors more readily upon transplantation into mice and are called brain tumor stem-like cells (BTSCs) also called brain tumor-initiating cells. These BTSCs have been shown to utilize a variety of mechanisms to preferentially survive therapy.

It is believed that our best hope for improved treatment lies in the development of therapies that selectively target this population. Successful therapeutic targeting of BTSCs requires improvements in our understanding of the signaling mechanisms that regulate their stem-like behavior. Similar to their normal stem cell counterparts, BTSCs are not randomly distributed throughout the tumor mass, but are concentrated in specific microenvironments. The microenvironments in which BTSCs reside, is gradually beginning to be understood to heavily influence the behavior of resident BTSCs. It follows that better characterization of the signaling events within these niches is central to understanding BTSC function.

Within certain brain tumors, particularly PDGF-induced gliomas, these BTSC microenvironments are observed in pseudopalisading necrotic regions and microvascular proliferating zones (also called perivascular niches).[2-4] Our current knowledge of the molecular mechanisms active within these niches in brain tumors is best understood in perivascular niche locations and will subsequently be the focus of this review. In the present work we review the current understanding of the various signaling pathways activated in the brain tumor perivascular niche (PVN) as well as recent evidence highlighting various inter-cellular cross-talk between the multitude of cell types located in the brain tumor PVN. Finally, we outline a model, based on the available evidence, to describe the potential signaling mechanisms operating in the PVN of brain tumors. Investigations of the cellular interactions and signaling pathways activated in the brain tumor PVN that potentially maintain BTSC properties may lead to the design of novel therapeutic strategies that can efficiently target the BTSC population.

The Tumor Perivascular Niche as a Haven for Brain Tumor Stem Cells

Adult neural stem cells (NSCs) are not distributed in random locations throughout the brain but are instead specifically concentrated in close proximity to blood vessels.[5] Indeed, the vasculature is an integral component of the major stem cell niches in the brain [the subventricular zone (SVZ) and subgranular zone (SGZ)], with a critical function of supporting stem cell proliferation and regeneration.[6,7] The importance of the vasculature to neural stem cells has been identified most clearly in the SVZ where in vivo, NSCs and their progenitors directly contact the vasculature. The vasculature appears critical for supporting NSCs and their progeny[6,7] by regulating the capacity of these cells to proliferate and differentiate. Emerging data suggest that this relationship extends to the microenvironment of brain tumors.

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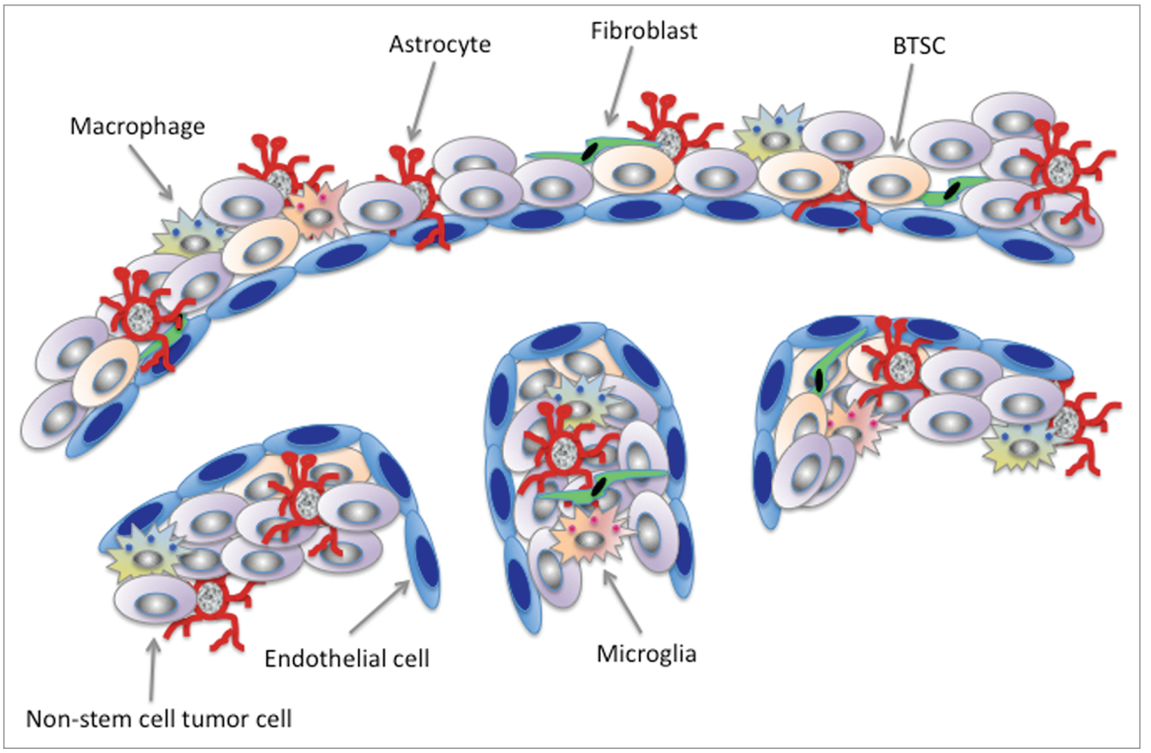

Figure 1. The brain tumor PVN is a heterogenous composition of cell types. Some of these include astrocytes, endothelial cells, macrophages, microglia, non-tumor initiating cells and brain tumor stem-like cells (BTSCs). BTSCs are intimately connected with endothelial cells, which are a source of niche-driven signals that help maintain BTSC properties. Astrocytes are closely aligned with endothelial cells but are also found widely dispersed throughout the tumor and signal to endothelial cells as ell as other stromal cells within the tumor microenvironment to support tumor progression. Therapies targeted to the PVN and individual contributions provided by stromal cells within the tumor microenvironment. More importantly, understanding the molecular signals that mediate interactions between multiple cell types is key to successfully finding therapies targeted to the PVN.

A characteristic feature of high-grade character in gliomas is the presence of microvascular proliferating structures.[1] These angiogenic entities represent regions of hyper-proliferative tumor stromal and endothelial cells.[8] The density of microvascular proliferation in human astrocytic brain tumors strongly correlates with patient survival.[9] In fact, the existence of microvascular proliferating structures is recognized as one of the early indicators of malignant progression and is currently a component that defines high-grade gliomas by the WHO.

The brain tumor PVN, defined as the area that borders angiogenic/tumor microvascular structures, is a prime location for BTSCs.[2-4] Recent data suggest that establishment of the brain tumor PVN facilitates expansion and differentiation of BTSCs. Indeed, the density of these PVN regions appeared to correlate with the number of BTSCs across several brain tumor subtypes including oligodendrogliomas and glioblastomas.[2]

A similar study identified PVN regions in medulloblastomas as prime locations for BTSCs that express both nestin and notch.[3] These nestin-expressing BTSCs preferentially survived radiotherapy relative to the tumor bulk to allow repopulation and regeneration of the tumor after therapy. Several reports identify a variety of mechanisms that mediate these radioresistant effects. A discussion of the mechanisms that mediate BTSC resistance to therapy is beyond the scope of this review, the interested reader is directed to the following reviews.[162,163] However, these studies highlight the growing recognition of the PVN as a critical participant in BTSC regulation.

Other Non-Tumor Perivascular Niche-Associated Cells

Currently, very little is understood about the structural organization of the PVN in brain tumors. In terms of the architectural make-up of cell types located in the PVN, we are only at the beginning stages of our understanding of their associated functions as they might relate to supporting resident BTSC populations localized to the niche. In addition to the BTSCs discussed above, the major cell types known to reside in the brain tumor PVN, are pericytes; immune cells including lymphocytes, macrophages and microglia; astrocytes, fibroblasts and endothelial cells that line the vasculature (**Fig. 1**). Each of these individual cell types makes distinct contributions to tumor progression by either contributing to the formation and stability of the microenvironment that supports the BTSC population or by promoting conditions within the niche that facilitates tumor progression.

Pericytes. Pericytes are reportedly derived from mesenchymal stem cells or neural crest cells.[11] They surround, stabilize and maintain the integrity of the walls of newly developed vasculature.[12,13] In the brain, pericytes have also been reported to be involved in immunological functions.[11] They tend to be largely

localized to microvessels where they are tightly associated with endothelial cells, separated only by a shared basement membrane. In addition, a codependent relationship appears to exist between pericytes and endothelial cells where they can each influence the mitotic activities of one another.[14] Pericytes express various markers some of which include, nerve/glial antigen 2 proteoglycan (NG2),[15,16] α-smooth muscle actin (α-SMA),[17] desmin[18] and platelet- derived growth factor receptor β (PDGFRβ).[19] However, none of these markers are absolutely specific for pericytes, their expression varies within specific tissues and developmental stages. Previous studies suggested a role for pericytes in microvascular proliferation in tumors. Pericyte recruitment has been identified to contribute to tumor vessel stabilization and survival of the vascular niche in pancreatic,[18] melanoma[20] and brain tumors.[10,21] In addition, in gliomas and other malignant human brain tumors, perivascular localized pericytes are believed to play a critical role in shaping the angio-architecture of the tumor vascular niche.[10,16]

Immune cells (macrophages). Immune cells are an additional component of the non-malignant cell populations located in the brain tumor microenvironment and include macrophages, lymphocytes and natural killer cells. As macrophages are the predominant immune-cell population in gliomas,[22,23] the discussion will focus on this population. In general, macrophages in the brain are referred to as microglia. Macrophages have been shown to reside in perivascular niche locations of the brain.[24] Historically, the presence of these immune cells in gliomas was postulated as a host defense mechanism to suppress dividing neoplastic cells[25] however, recent studies have identified glioma cells to play a direct role in recruitment of immune cells into tumors to support tumor growth.

Macrophages originate from $CD34^+$ bone marrow progenitors, which later mature into monocytes.[26] Monocytes subsequently differentiate into several cell types including macrophages, which constitute a significant proportion (5–20%) of the total cell population in brain tumors,[27] and can be as much as 50% in some tumors.[28] It has been suggested that BTSCs within the tumor microenvironment help facilitate tumorigenesis by driving an inflammatory environment in the BTSC vascular niche.[29] Macrophages can be localized to distinct locations following their recruitment into the tumor. They can be localized to the advancing tip of the tumor where they are involved in mediating tumor cell motility, they can be localized to stromal and PVN locations where they have been shown to be involved in promoting metastasis, or macrophages can be localized to perinecrotic areas where they promote angiogenesis.[30]

Macrophage recruitment to brain tumors is suggested to be a primary source of cytokines and other mediators that promote glioma proliferation and migration.[27] Monocyte chemoattraction protein-1 (MCP-1) has been implicated in driving macrophage recruitment to gliomas to promote glioma cell proliferation[31] and a recent report also suggests a similar role for MCP-3.[32] In addition to promoting glioma cell proliferation, macrophages have also been implicated in glioma tumor invasion, expansion and angiogenesis. For instance, upregulation of the membrane bound metalloprotease MMP-1, by tumor macrophages was required for glioma expansion.[33] Tumor infiltration of macrophages was strongly correlated with vascular density in human gliomas.[34,35] In a similar report, Tie-2 expressing monocytes (from which macrophages originate) were identified to function critically in the induction of angiogenesis in several glioma mouse models. Genetic deletion of Tie-2 expressing cells in vivo was found to substantially suppress angiogenesis and initiated significant tumor regression.[36] In a more recent study that utilized an orthotopic glioblastoma mouse model, macrophage recruitment was shown to play a role in the induction of brain tumor angiogenesis and tumor cell invasiveness.[37] Recruitment of macrophages in this study was dependent on the activity of HIF-1α. Some reports have also identified tumor suppressor effects of macrophages in brain tumors. For example bone marrow derived macrophages were demonstrated to bind and phagocytose T9 glioma tumor cells that expressed the membrane bound form of macrophage-CSF. Macrophage induced cytolysis of T9 glioma cells was shown to mediate survival of mice.[38] Tumor infiltrating macrophages were recently found to express the immune-modulatory nonclassical molecules HLA-G and HLA-E in a majorty of human GBMs analyzed. The expression of these molecules was suggested to function in tumor suppressor effects mediated by the macrophage population.[39]

Astrocytes. In the normal brain, astrocytes function as support cells as well as bonafide stem cells that express glial fibrillary acidic protein (GFAP).[40] Perivascular astrocytes are closely associated with the endothelium. This close association is tied to its critical function in the induction and maintenance of the blood brain barrier (BBB).[41] Astrocytes are known to regulate the activity of neural stem cells through contact as well as by secretion of diffusible signals.[42-44] At the molecular level, the specific role of reactive astrocytes in the perivascular niche of brain tumors is poorly understood. One study identified the localization of sonic hedge-hog (SHH) expressing reactive astrocytes in the perivascular niche of gliomas.[45] In this study, the levels of reactive astrocytes and Gli signaling were found to directly correlate with increasing grade of these gliomas. In addition, Gli signaling corresponded to the expression of SHH in the PVN of these tumors and the SHH expressing astrocytes were observed to be closely apposed to the perivascular nestin expressing stem cell-like population. Moreover, SHH/Gli signaling has been shown to be critical for BTSC self-renewal and is required for sustained growth and survival of gliomas.[46,47] In addition, tumor associated astrocytes play a role in glioblastoma cell invasion via activation of metalloproteinases.[48] Astrocyte elevated gene-1 (AEG-1) initially isolated from human fetal astrocytes,[49] was identified to be highly expressed in malignant gliomas and was demonstrated to mediate glioma invasion.[50] Furthermore, glioma stem cell-like/progenitor cells are known to express the SHH receptor Patched.[51] It is therefore plausible that perivascular reactive astrocytes identified in these giomas can mediate Gli activation in adjacent BTSCs. This would imply that reactive astrocytes in the glioma PVN may help maintain the stem cell properties of BTSC populations in these gliomas.

Endothelial cells. Endothelial cells are a core component of the BBB. They protect the brain from potentially harmful substances and also serve the nutritional demands of the CNS.

Endothelial cells originate from hematopoetic stem cells and some markers used to identify endothelial cells in gliomas include CD31, CD34 and CD36.[52]

Emerging data suggest that in addition to providing oxygen and nutrients to NSCs, endothelial cells are also important sources of diffusible niche factors, which regulate neural stem cell self-renewal and neurogenesis.[53] This mechanism appears to be conserved in brain tumors where endothelial derived factors appear to drive glioma cell proliferation[54] and self-renewal characteristics of perivascular BTSCs.[2,4] Signaling between endothelial cells and stem cells of the PVN is described below.

Fibroblasts. Fibroblasts have been shown to play an important role in tumor progression in other cancers.[55-57] Fibroblasts localized to the brain tumor microenvironment have been identified to promote the invasion of brain tumor cells. The expression of matrix metalloproteases is critical for breakdown of the ECM for tumor invasion. Fibroblasts were shown to produce and mediate activation of proMMP-2,[58] a metalloproteinase associated with increased invasiveness and malignant progression of gliomas.[59-61]

Intercellular Cross-Talk within the Tumor Perivascuar Niche

Communication between the multitude of cell types that exist in neurogenic niches of the normal brain is coordinated to help maintain neural stem cells and to preserve their potential to proliferate and differentiate. This logic may be extended to brain tumors as well.

Communication between endothelial cells and astrocytes. In the normal brain, astrocytes have traditionally been considered as support cells. The close association between endothelial cells and astrocytes enables effective communication between the two cell types to foster induction and maintenance of the BBB.[41] Crosstalk between perivascular astrocytes and endothelial cells can upregulate the expression of tight junction proteins,[62] GLUT1 and Pgp transporters[63,64] and BBB-associated enzyme activities of endothelial cells. These interactions ultimately enhance the stability of the vascular niche to support NSCs. Some studies suggest that endothelial cells may mediate growth and differentiation of perivascular astrocytes.[65] Whether similar intercellular communication between endothelial cells and perivascular astrocytes exists in the context of brain tumors is unclear. However, what we do know is that tumor development is associated with impaired BBB function and correlates with loss of astrocytic regulation of endothelial activity.[41] The extent to which it may contribute to tumor progression is not known.

Communication between endothelial cells and pericytes. Pericytes can communicate with endothelial cells through direct contact and also via pathways involved in paracrine signaling.[11] Pericyte-endothelial interactions have been reported in the regulation of blood flow and blood brain barrier functions.[14] During angiogeneis, pericytes can directly communicate with endothelial cells through direct interaction or through paracrine signals to affect the rate of endothelial cells differentiation and growth arrest.[14,66,67]

Communication between endothelial cells and BTSCs. Endothelial cells and NSCs are involved in molecular crosstalk that is mediated through direct interactions of NSCs with the vasculature[6] as well as through diffusible signals released from the vasculature.[53,68-70] Co-culture experiments of endothelial cells with embryonic or SVZ NSCs, spurred the self-renewal and proliferation of NSCs, which was mediated by diffusible signals from endothelial cells.[53] Pigment epithelium derived factor (PEDF), another diffusible signal identified to be secreted by endothelial cells has been proposed to regulate SVZ stem cell self-renewal.[70] Other factors include leukemia inhibitory factor (LIF)[65] and brain derived neurotrophic factor (BDNF).[68,71] Both factors are known to promote proliferation and/or differentiation in neurogenic niches of adults.

This intercellular communication appears to be conserved in the context of the brain tumor microenvironment. BTSCs residing in the tumor perivscular niche, like their normal stem cell counterparts, can engage in cell-to-cell communication with tumor endothelial cells. BTSCs were observed to home towards the vasculature where they initiated contact, aligning themselves along the entire length of the vasculataure.[2] The localization of BTSCs to the tumor vasculature puts them in an ideal position to respond to signaling cues from tumor endothelia. Soluble factors released from the endothelium have been shown to promote proliferation and self-renewal of BTSCs.[2] Moreover, increasing the quantity of endothelial cells and therefore the endothelial-derived factors enhanced the number of BTSCs, subsequently accelerating the initiation and growth of brain tumors in vivo.[2] More recently, nitric oxide has been identified as an endothelial-derived factor within the tumor perivascular niche of the PDGF-subset of gliomas that plays an important role in promoting BTSC properties. This endothelium-derived factor was shown to activate Notch signaling in a population of stem-like cells in a brain tumor mouse model. Activation of Notch signaling by NO accelerated the self-renewing capacity of BTSCs in vitro and enhanced the tumor initiating capacity of gliomas of the PDGF subtype in vivo.[4] These studies underscore the importance of the cross communication between the tumor vasculature and BTSCs, to support BTSC function.

However, the crosstalk between the vasculature and neural stem cells is by no means unidirectional. In fact, neural stem cells have been demonstrated to communicate to the vascular endothelium where they drive robust vascular tube formation by stimulating nearby endothelial cells to produce vascular endothelial growth factor (VEGF) and BDNF.[72] Like their normal stem cell counterparts, BTSCs can also modulate the activity of the tumor vasculature, which helps sustain them. Bao and coworkers demonstrated that human glioblastoma stem-like cells expressing elevated levels of VEGF could induce tumor endothelial cells to undergo angiogenesis.[73] In this study tumors derived from brain tumor stem cell-like populations showed greater levels of angiogenesis, necrosis and hemorrhage in comparison to their non stem cell-like counterparts expressing significantly lower levels of VEGF. This therefore indicates the bi-directionality of crosstalk between BTSCs and the vascular niche.

Signaling Pathways in the Brain Tumor Perivascular Niche

Our current knowledge of molecular pathways that are active in brain tumors are mainly from studies that describe these signaling pathways in the collective tumor microenvironment rather than in designated locations such as the PVN. Therefore, specific references will be made to those pathways that have specifically been identified, by reports, to be activated in the PVN and others will be described within the context of the overall tumor microenvironment.

The various cell types localized to the brain tumor PVN are involved in a multitude of signaling that may be coordinated to support the activity of BTSCs. These signaling pathways which include the SHH, PI3K/Akt, Wnt and the Notch signaling pathways have all been demonstrated to be activated in the microenvironemnt of brain tumors. As the aforementioned signaling pathways are known to regulate the self-renewal and proliferative properties of normal stem cells,[74] much research is focused on their potential involvement in regulating the stem-cell properties of BTSCs.

Sonic hedgehog signaling in the brain tumor PVN. Sonic hedgehog (SHH) is a secreted protein that plays a critical role in controlling fate of ventral cell types in the CNS during embryogenesis by mediating both cell proliferation and differentiation.[75-77] The binding of SHH to its receptor Patched releases tonic inhibition of Smoothened (SMO) by Patched. Smoothened release triggers activation of the Gli (named for their discovery in gliomas) family of transcription factors. These transcription factors subsequently induce translation of genes that function in maintaining the "stemness" of NSCs in the adult CNS.[78] SHH regulates the proliferation of astrocytic cells (type B cells) and transit amplifying cells (type C cells) in the adult SVZ.[36,79] Although GLI was first described in the context of gliomas,[80] its role in tumor formation has been studied in more detail in medulloblastoma, a primary brain tumor that arises in children.[81,82] Recent reports have helped to elucidate the mechanisms that mediate SHH induced proliferation and transformation of medulloblastomas.[83,84] A recent report has identified the transcription factor Yap1 expression in the PVN of medulloblatomas, which co-localized with stem-like markers and was identified to mediate SHH driven medulloblastoma formation.[83]

There have been several isolated studies exploring the importance of GLI and the broader SHH/PTCH/SMO/GLI cascade in gliomas. These studies have described the activity of SHH signaling within the collective tumor microenvironment. For example, *GLI* amplification occurs in a small subset of gliomas[85-87] and SMO inhibition by the drug cyclopamine reduces glioma cell proliferation.[46,51,88,89] Recent reports have suggested potential roles of the SHH pathway in glioma progression. Gli1 expression was closely correlated with pathological grades of human gliomas and RNAi knock-down of SHH/Gli signaling significantly suppressed glioma migration and invasion.[90] Using the RCAS/tva system in Gli reporter mice, Becher and colleagues demonstrated that sonic signaling was activated in mouse gliomas induced by PDGF overexpression. Immunohistochemical analysis of gliomas demonstrated elevated SHH protein expression localized to the glioma PVN regions and its expression was strongly correlated with increasing glioma grade.[45] These findings highlight the importance of the hedge-hog pathway in the microenvironment of brain tumors, however a specific role of the pathway in the PVN and potential effects on resident BTSC populations within the niche is yet to be described.

PI3K/Akt signaling in the brain tumor PVN. The PI3K/Akt pathway is frequently activated in gliomas ~70%.[91-93] Activation of this pathway is advantageous to the tumor as Akt promotes glioma proliferation, invasion and inhibits apoptosis and is thus critical for survival and growth of these tumors. Activation of the PI3K/Akt pathway and the expression of stem cell markers have been associated with aggressive behavior and tumor resistance. Studies in human gliomas demonstrate that alterations in components of Akt signaling at the DNA, RNA and protein levels correlates with poor prognoses in patients.[94] A number of other investigations that utilize genetic engineered mouse models of gliomas have shown that Akt activity contributes to glioma tumor formation and growth.[95-98] In addition, the expression of nestin and CD133 were shown to be strong prognostic factors for glioma malignancy.[99-101] Coincidentally, nestin-expressing BTSCs in mouse models of GBM are known to have high-levels of Akt pathway activity.[102]

In a recent study, BTSCs were demonstrated to be more dependent on Akt signaling than their matched non stem-cell counterparts. Subsequent inhibition of Akt by pharmacologic agents diminished BTSC capacity for neural stem cell formation, enhanced apoptosis and delayed tumor formation.[103] The significance of the role of PI3K/Akt signaling in facilitating resistance of PVN BTSCs to therapy, is highlighted by Hambardzumyan and coworkers, using mouse models of medulloblastomas. In this study BTSCs (identified by the expression of nestin and Notch) were found to preferentially survive radiation therapy relative to the non-stem cell populations within the tumor by activating the PI3K/Akt signaling pathway in response to radiation. These BTSCs preferentially survived by enhancing their expression of nestin and arresting instead of undergoing apoptosis as the bulk of tumor cells did. Following a temporary period of arrest (72 hrs), these BTSCs reentered the cell cycle to proliferate and reconstitute the tumor following therapy.[3] The importance of AKT signaling in PDGF-induced gliomas has also been demonstrated in preclinical trials in glioma-bearing mice by blocking the activity of mTOR using the rapamycin analog temsirolimus.[104]

Notch signaling in the brain tumor PVN. Notch signaling is highly conserved across evolution and is critically important for directing patterning during development and plays a key role in stem cell proliferation and self-renewal.[105] Notch ligands and receptors are single-pass transmembrane proteins that mediate Notch signaling.[106] Notch signaling involves the binding of the Notch ligands (Delta-like 1, 3 & 4 and Jagged 1 & 2) to Notch receptors (Notch 1–4) in a juxtacrine signaling mechanism. Ligand-receptor interaction induces a series of cleavages the final of which involves gamma secretase cleavage of Notch intracellular domain (NICD). NICD translocates to the nucleus where the binding to CSL transcription factors culminates in neural stem cell proliferation and suppression of their differentiation[107,108]

among many other functions. Notch is required for neural progenitors both in vitro and in vivo[109] and it is essential for the maintenance of neural stem cells.[110,111]

With regards to cancer, a substantial amount of data has linked activation of the Notch pathway to tumor development in many types of cancers[112] including brain tumors. The expression of Hey1 (a downstream target of Notch) was correlated with tumor grade and survival of human GBM patients.[113] Notch-1, along with its receptors Jagged-1 and delta- like-1, play an integral role in high-grade glioma and medulloblastoma formation.[114-117] Notch was identified to drive BTSC activity in medulloblastomas.[118] Pharmacological blockade of the notch pathway diminished nestin- and CD133-positive populations as well as the side population (SP) within medulloblastoma cells and decreased their tumor initiating capacity in vivo. Notch activation was also shown to enhance the expression of nestin in these medulloblatoma cells.[118] The link between Notch signaling and BTSC function in gliomas was established in a related study that identified CSL binding sites for Notch in the promoter region of human nestin, implying regulation of nestin by the Notch pathway. In this investigation Notch signaling was shown to increase the expression of nestin and in combination with Kras, induced proliferative lesions in the NSC enriched periventricular regions in a glioblastoma mouse model.[119] In addition, activated Notch signaling in a human glioma cell line enhanced the formation of neurosphere-like colonies[120] and Notch signaling was required for Id-4 induced neural stem-like properties of Ink4aArf$^{/-}$ astrocytes.[117]

We have recently identified a critical role of the notch pathway in driving the stem cell-like characteristics of BTCSs which reside in the glioma PVN.[4] Perivascular nestin- expressing BTSCs co-expressed Notch-1 and activation of Notch mediated through nitric oxide signaling was shown to enhance the tumor initiating capacity of PDGF-induced glioma primary cultures.[4]

Notch signaling within the tumor vascular microenvironment has also been shown to regulate tumor angiogenesis in several cancers.[121-123] Delta-like4 (DLL4) expression was shown to be upregulated in tumor cells and tumor endothelial cells of human glioblastoma. Using a mouse model of glioblastoma it was demonstrated that DLL4 expression by tumor cells activated notch signaling in endothelial cells to drive tumor angiogenesis.[124] In addition, anti-DDL4 treatment of tumor cells in a glioblastoma model inhibited growth of glioblastomas by paradoxically enhancing growth of non-functional tumor vasculature.[125] Collectively, these studies have highlighted the importance of the Notch pathway in tumor development, and suggest that selective targeting of the Notch pathway may be an effective way to target the brain tumor microenvironment.

Nitric oxide (NO)/cGMP signaling in the brain tumor perivascular niche. NO is a highly reactive free radicle with a half-life of approximately 2–5 secs.[126] It is a highly lipophilic and diffusible molecule, which can readily disperse from its site of synthesis to traverse multiple cell membranes en route to its final target. NO is generated from a family of NADPH-dependent enzymes called nitric oxide synthases (NOS) from the terminal guanido nitrogen atom of L-arginine. The three major NOS isoforms are neuronal (nNOS), inducible (iNOS) and endothelial NOS (eNOS). In general nNOS and eNOS are expressed constitutively in neurons and endothelial cells respectively, but can be expressed by other cells. Their activity depends on the levels of intracellular calcium to produce approximately nanomolar amounts of NO. Expression of iNOS in contrast, is induced, and its expression is generally not affected by levels of intracellular calcium.[127] The major NO signaling pathway involves production of cyclic guanosine-3',5'-monophosphate (cGMP) by way of activation of soluble guanylyl cyclase, the major receptor for NO and its downstream effector, protein kinase G (PKG).

We have recently demonstrated signaling by the NO/cGMP pathway in the perivascular niche of the PDGF subset of gliomas.[4] We show that NO activates Notch signaling in a population of perivasular localized brain tumor stem-like cells that express nestin and notch. This is consistent with previous reports where NO was identified to drive activation of the Notch pathway in cholangiocarcinomas.[128] Our data demonstrates that activation of Notch signaling in perivascular BTSCs by NO released from the tumor endothelium enhanced BTSC-like characteristics and accelerated tumor formation in mice. This data identifies components of the NO-cGMP pathway as potential targets for therapy and suggests that changes of brain tumor cells towards BTSC-like features can be induced by the microenvironment.

Other brain tumor stem cell signaling pathways that may or may not contribute to perivascular niche biology. In addition to the pathways mentioned above, there are several other signaling pathways known to be important regulators of BTSC activity. These include the Bone morphogenetic protein/Smad (BMP/Smad) signaling and Wnt signaling pathways. Nonetheless, although the assertion can be made that these pathways are likely active in the brain tumor PVN specifically, this remains to be demonstrated.

BMP/Smad signaling. Bone morphogenetic proteins (BMPs) are members of the TGF-β/Smad family of growth factors that regulate a variety of biological processes including bone and cartilage formation and embryonic stem cell self-renewal and differentiation.[129] Binding of BMPs to their cognate receptors BMPRs elicits the downstream effects of BMPs, which involves activation of Smad proteins. BMPs play a critical role in the regulation of NSC proliferation and apoptosis, and usually promotes NSC differentiation.[130] BTSCs were identified to express BMPRs and therefore retained the capacity for regulation by BMPs. Treatment of BTSCs with BMPs inhibited proliferation of BTSCs and induced their differentiation to astroglial and neuron-like cell fates, which diminished the capacity of BTSCs to initiate tumors in vivo.[131] A similar report by Lee and co workers demonstrated differentiation of BTSCs following BMP treatment. However, a subset of BTSCs that did not express the appropriate BMPR1B receptor showed enhanced proliferation in response to BMP treatment instead of differentiation. Forced expression of BMPR1B sensitized this subset of BTSCs to BMP-induced differentiation[132] suggesting that the expression pattern of BMPR may dictate the response of BTSCs to BMP treatment.

A more recent study demonstrated that knockdown of TRRAP—an adaptor protein with homology to PIKK

kinases—could suppress BTSC proliferation and induce their differentiation to an astroglial lineage. These effects of TRRAP were mediated by suppression of cyclin A2 gene expression and enhanced BMP/Smad signaling.[133,134]

Wnt signaling. The Wnt pathway is involved in several key developmental processes such as proliferation, stem cell maintenance, pattern formation and differentiation.[135] Wnt signaling has been shown to play a role in SVZ progenitor cell proliferation and neurogenesis in neonatal and adult mice[136,137] and to regulate stem cell fate and differentiation in vivo.[138] Wnt signaling was initially implicated in contributing to medulloblastomas.[139,140] Mutations in downstream mediators of Wnt signaling, such as Axin, β-catenin and APC were found in sporadic medulloblastomas,[141-143] and nuclear localization of β-catenin, which is indicative of constitutive Wnt signaling, has also been found.[144]

Moreover, DKK-1, an endogenous antagonist of the Wnt pathway was reported to be epigenetically silenced in medulloblastomas and its reactivation was found to enhance apoptosis and induce a 60% reduction in tumor growth.[145]

Negative regulators of the Wnt pathway are frequently hypermethylated in glioblastomas.[146] Ectopic expression of the Wnt negative regulator, DKK-1 in the human glioma U87MG cell line sensitized these cells to chemotherapy-induced apoptosis.[147] In addition, several components of the Wnt pathway were found to be upregulated in gliomas, including Wnt 5A and Frizzled 9, a receptor of the canonical Wnt pathway[148-150] The Wnt pathway has also been demonstrated to mediate cell proliferation and the invasive capacity of gliomas. Immunohistochemical analysis of 45 human astrocytomas demonstrated an upregulation of core components of the Wnt pathway. Suppression of these components significantly decreased cell proliferation and invasion and delayed tumor formation in mice. This effect of Wnt suppression on gliomas was associated with disruption of the PI3K/Akt pathway[151] suggesting cross communication between these two signaling axes.

Therapeutic Targeting of the Brain Tumor Perivascular Niche Complex

According to the cancer stem cell hypothesis, BTSCs are the engines that drive brain tumor progression and recurrence. Therefore, effective targeting of this population is critical to all efforts designed to eradicate brain tumors. Since BTSCs are known to reside in the PVN regions of these tumors, one promising strategy involves targeting intracellular pathways that are active in the brain tumor PVN, pathways which promote BTSCs self-renewal, proliferation and migration. These signaling pathways include SHH, PI3K/AKT, Notch and more recently the NO/cGMP signaling pathway. Targeting these pathways with inhibitors have all been demonstrated to suppress glioma progression and sensitize brain tumors to therapy.[3,89,118,152,153] Additional pathways such as DNA damage checkpoint pathways (Chk1&2), Wnt and BMP/Smad that regulate the activity of brain tumor cells are also important, and targeting these pathways have been demonstrated to suppress brain tumor growth in experimental and preclinical models.[131,133,154] However, it is unknown whether these pathways are active in the PVN of brain tumors.

Targeting endothelial cells of the tumor vasculature can be an alternative approach used to subvert BTSC function. Studies have shown that disrupting the vascular niche microenvironment in which BTSCs reside, and which is critical for their maintenance might be a viable approach to targeting BTSCs.[2,155] One might imagine that destruction of the vascular niche likely eliminates the supportive environment the vasculature provides to BTSCs. Indeed, Bevacizumab (Avastin), a monoclonal antibody targeted against VEGF-A and Cediranib (AZD2171), a small molecule inhibitor of VEGF, have been used in the clinic with some success.[156-158] In experimental models, targeting the brain tumor vascular niche with the use of anti-angiogenics has shown some promise. The anti-angiogenic monoclonal antibody against VEFGR-2, DC101, markedly suppressed experimental malignant glioma growth.[159] The suppression of tumor volumes and microvessel density of tumor-bearing mice, relative to control animals, corresponded with decreased tumor cell proliferation and increased apoptosis. The authors of the study observed an increase in tumor invasive features from DC101 monotherapy, which was suppressed with combined EGFR blockade.[160] Another study that utilized PTK787, an inhibitor of VEGFR and PDGFR tyrosine kinases, led to significant reductions in tumor volumes and vessel density.[161]

Although suppression of the tumor-associated effects of many of these studies is ascribed to suppression of angiogenesis, it is conceivable that adverse effects on the BTSC populations might have also contributed to these overall effects of tumor suppression. Examination of the BTSC populations before and after treatment in these tumors may shed some light on this possibility. Indeed, work by Calabrese and co workers demonstrated that treatment of mice bearing orthotopic xenografts of human glioblastomas cells with bevacizumab, significantly diminished the population of perivascular localized BTSCs (with little impact on the actual tumor cells) and suppressed tumor growth rate.[2] In addition, Folkins et al. showed, using a C6 rat glioma model, that anti-angiogenic treatment in combination with cytotoxic compounds, diminished the BTSC populations as well as their neurosphere forming capacities.[155] These studies strongly suggest that current anti-angiogenic treatments may already be targeting the BTSC populations in brain tumors.

Conclusion

The tumor PVN microenvironment is composed of a complex array of cell types, each with individual contributing roles to play in the maintenance of the tumor. This reality has far- reaching implications for therapies designed to target the PVN. These therapies will have to consider the complex milieu of cellular elements within the PVN and the signals that facilitate communication among resident cell types. Approaches designed to antagonize the various combinations in which these signals may interact, will be key to successfully targeting the PVN for therapy.

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